

Prolonged activation of ASIC1a and the time window for neuroprotection in cerebral ischaemia

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Acid-sensing ion channels (ASICs), newly discovered members of epithelial Na⁺ channels/degenerin superfamily, are widely distributed throughout the mammalian peripheral and central nervous system and have been implicated in many physiological and pathophysiological processes. We have recently shown that activation of calcium-permeable ASIC1a is involved in acidosis-mediated, glutamate independent, ischaemic brain injury. In this study the neuroprotective time window for ASIC1a blockade in a mouse model of focal ischaemia is examined and the role of acidosis *per se* addressed by continuous pH measurements in penumbral cortex and post-ischaemic alkalization of brain. The effects of NMDA receptor blockade and ASIC1a blockade were compared. Specific ASIC1a blockade by the tarantula toxin psalmotoxin, PcTX, administered intracerebroventricularly as late as 5 h after 60 min of transient middle cerebral artery occlusion (MCAO) reduced infarct volume by >50%; the protection persisted for at least 7 days. Protection was also demonstrated after permanent MCAO. In penumbral cortex alkaline pH preceded acid pH and infarction. Attenuating brain acidosis by NaHCO₃ or blocking ASIC1a with PcTX were both protective. NMDA blockade produced additive neuroprotection and the presence of PcTX prolonged the time window of effectiveness of NMDA blockade. Neuroprotection by PcTX was also achievable by intranasal administration. These findings further suggest that ASIC1a is a novel molecular target involved in ischaemic brain injury. Post-ischaemic administration of an ASIC1a blocker may prove to be an effective neuroprotective strategy for stroke patients.

Keywords: acidosis; acid-sensing ion channels; ischaemia; neuroprotection

Abbreviations: ASIC = acid-sensing ionic channels; CBF = cerebral blood flow; i.c.v. = intracerebroventricularly; i.n. = intranasal; MCAO = middle cerebral artery occlusion

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Introduction

In the last decades enormous effort has been made in an attempt to attenuate stroke injury. Despite the attractiveness of the excitotoxicity hypothesis, all clinical trials using glutamate antagonists as protective agents against ischaemia have failed. This may be partially due to other injury pathways and non-excitotoxic processes involved in the complex mechanisms triggered by the lack of blood supply in brain. In this regard, the role played by acid-sensing ion channels (ASICs) in cerebral ischaemia has been recently underlined (Xiong *et al.*, 2004; Benveniste and Dingledine, 2005; Gao *et al.*, 2005).

ASICs belong to the degenerin/epithelial Na⁺ channel family of amiloride-sensitive cation channels. Various ASIC subunits form homomultimeric and heteromultimeric

channel complexes that vary in their expression within organs and are activated at different pH values (Krishtal, 2003). Of six ASIC subunits cloned, ASIC1a, ASIC2a and ASIC2b are expressed in brain neurons (Waldmann *et al.*, 1996; Lingueglia *et al.*, 1997; Waldmann *et al.*, 1997b; Duggan *et al.*, 2002). ASIC1a, which has a pH for half maximal activation (pH_{0.5}) of 6.2 (Waldmann *et al.*, 1997a) allows the passage of both Na⁺ and Ca²⁺ ions into the cells (Lingueglia *et al.*, 1997; Waldmann *et al.*, 1997a; Wu *et al.*, 2004; Xiong *et al.*, 2004; Yermolaieva *et al.*, 2004) and is the most likely ASIC involved in physiological (Wemmie *et al.*, 2002; Wemmie *et al.*, 2003, 2004; Xiong *et al.*, 2004) and pathological conditions (Obrenovitch *et al.*, 1990; Deitmer and Rose, 1996; Li and Siesjo, 1997; Simpson *et al.*, 1997;

Diarra *et al.*, 1999; Allen and Attwell, 2002). Our recent studies have demonstrated that activation of ASIC1a is largely responsible for acidosis-mediated, glutamate independent neuronal injury in ischaemic brain, disclosing a potential novel therapeutic target for stroke patients (Xiong *et al.*, 2004).

The main objective of the present study is to determine the time window for neuroprotection by ASIC1a blockade in brain ischaemia modelled in mouse and to confirm the central role of acidosis in ischaemic brain injury by alkalization of post-ischaemic brain and by direct, continuous measurement of pH in penumbral cortex and in the evolving ischaemic core.

Finally, since it has been recently shown that the NMDA/ Ca^{2+} -calmodulin-dependent protein kinase II (CaMKII) cascade is functionally coupled to ASICs and contributes to acidotoxicity during ischaemia (Gao *et al.*, 2005), the effect of complementary blockade of ASICs and NMDA channels was also examined.

Material and methods

Experimental groups

Male C57B mice (Charles River) weighing 25–30 g were housed under diurnal lighting conditions (12 h darkness and 12 h light). $N = 5$ group sizes were used. Additional animals were later added to experimental groups for confirmation. Experiments were performed according to the international guidelines for animal research. All experiments were performed in accordance with the American animal protection legislation and approved by the Institutional Animal Care and Use Committee of Legacy Research.

Focal ischaemia

Transient focal ischaemia was induced by suture occlusion of the middle cerebral artery (MCAO) in male mice anaesthetized using 1.5% isoflurane, 70% N_2O and 28.5% O_2 (Xiong *et al.*, 2004). Ischaemia was induced by introducing a coated filament (6.0; Doccol) from the external carotid artery into the internal carotid artery and advancing it into the circle of Willis to the branching point of the left MCA, thereby occluding the middle cerebral artery (Longa *et al.*, 1989). Achievement of ischaemia was confirmed by monitoring regional cerebral blood flow (CBF) in the area of the left middle cerebral artery. CBF was monitored through a disposable microtip fibre optic probe (diameter 0.5 mm) connected through a Master Probe to a laser Doppler computerized main unit (PF5001, Perimed, Sweden). The microtip was attached to the skull of the mouse through cyanoacrylate glue. Data were analysed by means of dedicated software (PSW Perisoft 2.5) (Kawano *et al.*, 2006). Animals that did not show a CBF reduction of at least 70% were excluded from the experimental group (Bano *et al.*, 2005), as were animals that died after ischaemia induction. Rectal and temporalis muscle temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with a thermostatically controlled heating pad and lamp. Rectal temperature was monitored every 30 min for 6 h after MCAO in all the animals. No significant differences were found between treated and untreated animals. All surgical procedures were performed under an operating stereomicroscope. To induce permanent middle cerebral artery occlusion (MCAO) the same

procedure was used except that the coated filament was not removed until the animal was killed.

Evaluation of the infarct volume

Animals were killed using isoflurane 24 h or 7 days after ischaemia. Brains were quickly removed, sectioned coronally at 1 mm intervals, and stained by immersion in the vital dye (2%) 2,3,5-triphenyltetrazolium hydrochloride (TTC) (Bederson *et al.*, 1986). The infarct volume was calculated by summing infarction areas of all sections and multiplying by slice thickness. The percentage of the infarct was calculated by dividing the infarct volume by the total ipsilateral hemispheric volume, this ratio was then multiplied by 100 (Pignataro *et al.*, 2004).

Experimental protocol

Drugs were administered intracerebroventricularly (i.c.v.), intravenously (i.v.), intranasally (i.n.), and/or intraperitoneally (i.p.). PcTX was obtained as the venom of the South American tarantula *Psalpopus cambridgei* (Escoubas *et al.*, 2000). Animals were assigned in treatment groups in a blinded fashion and infarct volume was measured in a blinded manner.

Vehicle (aCSF; 0.5 μl), PcTX solution (0.5 μl) and NaHCO_3 (0.5 μl) were i.c.v. injected using a 1 μl Hamilton syringe, at different times following the MCAO, into the lateral ventricle, 0.75 mm laterally, 0.1 posteriorly and 2.1 mm deep relative to the bregma. PcTX was infused 15 min, 1, 2, 2.5, 3, 5, 6 or 12 h after ischaemia induction, at a dosage of 500 ng/ml (~ 10 ng/kg). In order to establish a dose–response, PcTX was also injected 1 h after MCAO at the dosage of 50 ng/ml (1 ng/kg) and 16 (0.3 ng/kg). NaHCO_3 was administered 1, 4 or 5 after MCAO, at the concentration of 125 mg/ml (2.5 mg/kg). Different concentrations of NaHCO_3 , e.g. 0.125 (0.025 mg/kg) and 12.5 mg/ml (0.25 mg/kg) were also i.c.v. infused 1 h after MCAO.

For i.v. injection, vehicle (saline; 100 μl), PcTX solution (100 μl , 500 ng/ml or 5 mg/ml, 2 or 20 $\mu\text{g/kg}$) and NaHCO_3 (100 μl , 125 or 250 mg/ml, 2.5 or 5 mg/kg) were administered into the femoral vein 1 h after MCAO.

Vehicle (aCSF; 50 μl) and PcTX solution (50 μl , 500 ng/ml, 10 ng/kg) were administered i.n. 1, 4 and 5 h after MCAO. A total volume of 50 μl was delivered with an Eppendorf pipette in 10–12 μl drops to alternating nostrils every 2 min.

Vehicle (saline; 1 ml) and memantine (1 ml, 10 mg/kg) were administered i.p. 15 min, 1 or 3 h after MCAO. Furthermore, different concentrations of memantine, 1, 10, 30 and 100 mg/kg, were used 1 h after MCAO.

Individuals blinded to treatment groups performed all manipulations and analyses.

Cerebral pH measurement after ischaemia

For continuous measurement of pH in the brain, a fibre optic pH micro system (pHOptica, WPI) was used. This system has a pH range between 5 and 9, and the resolution is ± 0.003 units. A glass fibre with its pH-sensitive tip (140 μm OD) was stereotactically implanted into the brain immediately after the MCAO at the following coordinates from the bregma: 2.4 mm laterally, 0.1 posteriorly and 2.2 mm deep. pH was continuously measured for 6 h. The instrument was calibrated with standard solutions (pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) before and after the experiments. To mimic the brain environment the standard solutions were pre-warmed at 37°C and the calibration was performed in the

absence of light. Changes in pH values were evaluated after PcTX and NaHCO_3 i.c.v. or i.v. administration.

Statistical analysis

Values are expressed as means \pm SD. Statistical analysis was performed with ANOVA followed by the Newmann–Keul test. Statistical significance was accepted at the 95% confidence level ($P < 0.05$).

Results

Neuroprotective time window of PcTX in focal cerebral ischaemia

For the mouse ischaemia model, the left middle cerebral artery was occluded for 60 min, followed by 24 h of reperfusion. To determine the protective time window for ASIC1a blockade, the specific ASIC1a blocker PcTX or vehicle was infused i.c.v. at 15 min, 1, 2, 2.5, 3, 5 and 6 h after MCAO and the percentage of the ipsilateral hemisphere infarct in the vehicle-injected animals was 50.57 ± 5.2 , 48.09 ± 3.8 , 42.69 ± 5.8 , 50.89 ± 7.0 , 50.63 ± 5.2 , 50.59 ± 4.7 and 50.64 ± 8.7 , respectively ($n = 5$ for each group, Fig. 1A). However, i.c.v. injection of PcTX (0.5 μl , 500 ng/ml total protein, ~ 10 ng/kg) at 15 min ($n = 6$), 1 h ($n = 5$), 2 h ($n = 10$), 2.5 h ($n = 5$), 3 h ($n = 11$) or 5 h ($n = 8$) after MCAO significantly reduced the percentage of the infarct to 25.79 ± 10.7 , 23.18 ± 12.0 , 19.77 ± 10.0 , 26.42 ± 8.6 , 30.95 ± 8.9 or 34.22 ± 4.6 , respectively (Fig. 1A). The original group sizes were $n = 5$, which resulted in identical statistical significance ($P < 0.05$). PcTX injection at 6 h did not reduce the infarct volume ($n = 5$, infarct volume = $51.02 \pm 9.6\%$). Inactivated (boiled) PcTX was not protective when it was injected at 1 h (infarct volume = $46.4 \pm 3.2\%$, $n = 5$; Fig. 1A). The mortality rate in the vehicle-treated animals and in the PcTX-treated animals was 14.6 and 12.7%, respectively ($P > 0.05$). Because of insufficient CBF reduction, 18.7% of the animals, equally distributed in the two groups, were also excluded from the experimental groups.

To evaluate the dose-dependent effect of PcTX, diluted PcTX (10 and 30 times) was also used. A significant reduction of infarct volume (by $\sim 40\%$) was observed when PcTX was used at a total protein concentration of 1 ng/kg (0.5 μl at 50 ng/ml, $n = 5$, % of infarct = 23.6 ± 2.3), while a further decrease of dose to 0.3 ng/kg (0.5 μl at 16.5 ng/ml) was not protective ($n = 5$, % of infarct = 43.9 ± 3.1 ; Fig. 1B). A mortality rate of 10.7% occurred in this set of experiments, with no significant difference between the control group and the treated groups. Furthermore, 4.1% of mice were excluded from the experimental group because CBF reduction did not reach 70%.

To determine whether PcTX prevented cell death or only delayed it, we extended our observations of infarct volume to 7 days after the ischaemia, with PcTX administration 1 h after MCAO. Similarly, PcTX decreased infarct volume by $\sim 50\%$ (23.9 ± 5.9 versus $53.4 \pm 8.6\%$, $n = 6$; Fig. 2A) when

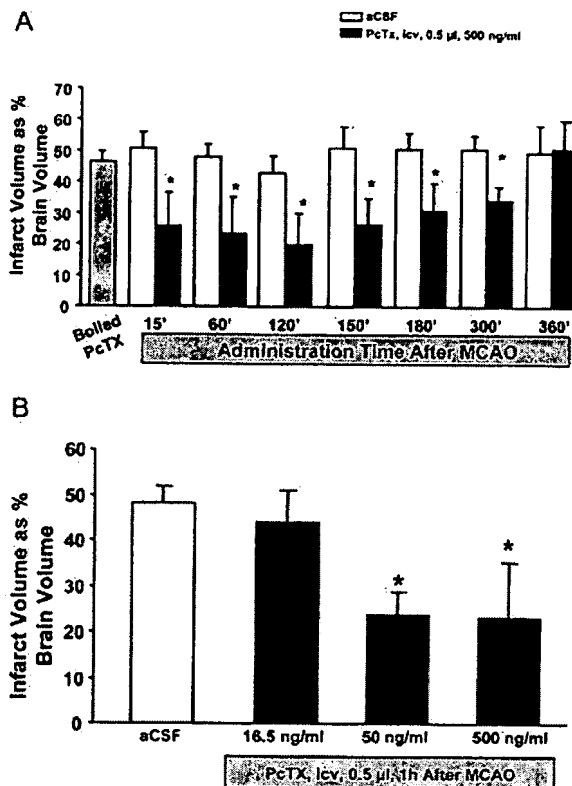


Fig. 1 (A) Effect of PcTX on infarct volume induced by 60 min of MCAO, evaluated 24 h after induction of ischaemia. Each column represents the mean \pm SD of the percentage of the infarct volume compared with the ipsilateral hemisphere. * $P < 0.05$ versus boiled-PcTX-treated mice and vehicle-treated group. **(B)** PcTX dose-response curve. Data are presented as mean \pm SD of the percentage of the infarct volume compared with the ipsilateral hemisphere. * $P < 0.05$ versus vehicle-treated group.

brains were examined at 7 days. The mortality rate at 7 days was 31.5%, equally distributed between vehicle-treated mice and PcTX-treated mice. Because of insufficient CBF reduction 5.3% of animals were excluded. Pronounced reduction of infarct volume by PcTX (injected 1 h after occlusion) was also obtained in a permanent model of cerebral ischaemia (12.3 ± 3.7 versus $57.6 \pm 11.8\%$, $n = 5$; Fig. 2B). No statistical significance was found in the protection by PcTX in temporary MCAO and permanent MCAO models (23.18 ± 12.0 versus 12.3 ± 3.7). Again, because of insufficient CBF reduction, 8.3% of animals were excluded from the experimental group and 8.3% of the animals died after surgery. There was no difference between PcTX-treated and vehicle-treated animals.

Although ASIC1 blockade via i.c.v. administration provided a prolonged protective time window, this route of delivery will be difficult to achieve clinically. For this reason, we determined whether this peptide is still effective if administered systemically. PcTX was therefore administered through the femoral vein (i.v.) and through the nasal cavity.

PcTX venom administered i.v., 1 h after ischaemia, was not able to modify the infarct volume (% of infarct volume = 49.1 ± 12.3 with PcTX injection versus 47.9 ± 4.2 with saline injection, $n = 5$ in each group). But i.n. administration of PcTX did reduce the infarct volume by $\sim 50\%$ when it was administered 1 h after MCAO ($n = 5$, infarct volume = $21.4 \pm 5.4\%$). The same protection was recorded when PcTX was administered i.n. 4 h after MCAO ($n = 5$, infarct volume = $23.8 \pm 3.9\%$). In vehicle-treated animals, the percentage of the infarct volume was 53.4 ± 8.1 ($n = 5$; Fig. 2C). Twelve per cent of mice died after surgery and 8% of animals did not reach sufficient CBF reduction. Again, no difference occurred between treated and vehicle control groups.

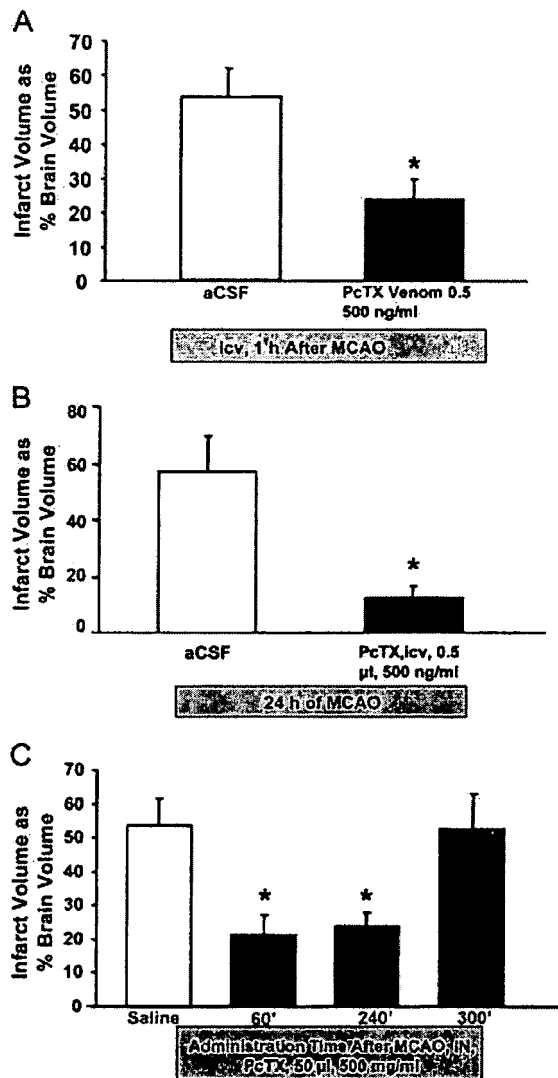


Fig. 2 Effect of PcTX on infarct volume at 7 days of reperfusion after 60 min MCAO (A), 24 h after permanent MCAO (B) and after i.n. administration of PcTX (C). Each column represents the mean \pm SD of the percentage of the infarct volume compared with the ipsilateral hemisphere. * $P < 0.05$ versus vehicle-treated group.

Our recent studies have demonstrated that ASIC1a blockade may provide additional neuroprotection in the presence of NMDA receptor antagonist memantine (Xiong et al., 2004). To further investigate the potential interaction between NMDA and ASIC1a blockade on infarct volume, memantine was administered before or after PcTX administration. The dosage of PcTX (500 ng/ml) and memantine (10 mg/kg) used in these experiments was the maximum effective dosage, as previously determined by us (for memantine, data not shown). A further reduction of the infarct volume was observed when memantine (10 mg/kg) was administered i.p. 30 min ($n = 5$, infarct volume = $15.9 \pm 5.0\%$) and 1 h ($n = 5$, $15.6 \pm 4.1\%$) but not 1.5 h after MCAO, in PcTX-treated mice (10 ng/kg, injected 15 min after MCAO). Similarly, an additional protection was observed when PcTX was administered 3 or 5 h after MCAO ($n = 5$, infarct volume 16.1 ± 4.1 and $n = 5$, infarct volume = $10.8 \pm 2.6\%$, respectively), but not 6 h, in memantine-treated mice (10 mg/kg, 15 min after MCAO; Fig. 3A). Because of insufficient CBF reduction 13.3% of mice were excluded and a mortality rate of 11.6% occurred in this

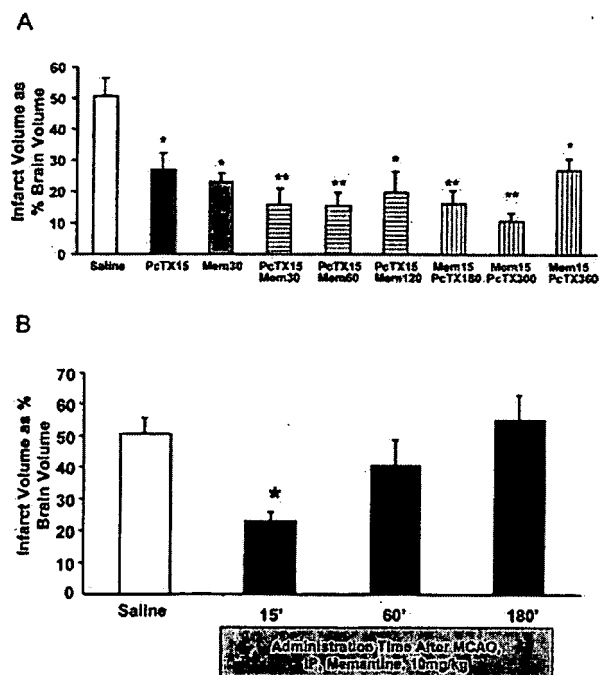


Fig. 3 (A) Combined effect of i.c.v. PcTX and i.p. memantine on infarct volume induced by 60 min of MCAO, evaluated 1 day after induction of ischaemia. Each column represents the mean \pm SD of the percentage infarct compared with the ipsilateral hemisphere. The numbers listed after PcTX and memantine indicate the time, in minutes, after ischaemia induction. * $P < 0.05$ versus control group; ** $P < 0.05$ versus control group, PcTX-treated mice and memantine-treated mice. (B) Memantine's time window (1 ml, 10 mg/kg). Each column represents the mean \pm SD of the percentage of the infarct compared with the ipsilateral hemisphere. * $P < 0.05$ versus vehicle-treated group.

set of experiments. There was no difference between treated and control mice.

A prolonging of the memantine therapeutic time window was observed when it was administered following PcTX administration. Memantine alone administered i.p. 1 h after MCAO was not able to produce a neuroprotective effect (infarct volume = $40.3 \pm 8.5\%$ with memantine versus $50.6 \pm 5.2\%$ with saline; $n = 5$ in each group; Fig. 3B). This finding is consistent with various published studies (Wahlgren and Ahmed, 2004). To verify if the limited therapeutic time window is due to the memantine concentration used, the effects of different dosages (1, 10, 30 and 100 mg/kg of the memantine) were evaluated. In all cases, when memantine was administered 1 h after MCAO, we did not observe any significant reduction of the infarct volume. In the vehicle-treated animals ($n = 5$), the percentage of the infarct volume was 50.6 ± 5.6 , while in animals treated with 1, 10 or 30 mg/kg memantine ($n = 5$ for each group) the infarct volume was 55.4 ± 12.7 , 41.0 ± 8.3 and 43.4 ± 9.3 , respectively (all non-significant). Mortality rate in 1, 10 and 30 mg/kg groups was 12.0%, similar to the other treatment groups. However, all mice died with the highest dosage of memantine (100 mg/kg, $n = 5$; data not shown).

On the contrary, i.p. administration of 10 mg/kg memantine 1 h after MCAO provided additional neuroprotection when administered in PcTX-treated animals (15.6 ± 4.1 , as compared to 41.0 ± 8.3 for memantine alone or to 23.18 ± 13.0 for PcTX alone, Fig. 3A). A similar phenomenon was reported for the association between memantine and the 2-adrenoreceptor agonist clenbuterol (Culmsee *et al.*, 2004).

Neuroprotection by i.c.v. injection of bicarbonate

It has been established that the neuroprotective effect by PcTX venom is due to the blockade of ASIC1a (Xiong *et al.*, 2004). In ischaemia, pH of brain tissue falls to a range which activates ASIC1a (Xiong *et al.*, 2004). If the fall of pH is indeed the neurochemical feature responsible for ischaemic injury, alkalinization of the ischaemic brain tissue should also be able to attenuate the brain damage. To test this hypothesis a sodium bicarbonate solution was i.c.v. infused at different concentrations and at different times (1, 4 or 5 h) following MCAO. The effective concentration of bicarbonate was established to be 2.5 mg/kg ($n = 5$, infarct volume = $26.2 \pm 5.8\%$). Lower concentrations, for example, 0.25 mg/kg ($n = 5$, infarct volume = $52.4 \pm 2.7\%$) or 0.025 ($n = 5$, infarct volume = $54.1 \pm 3.5\%$) did not have any effect on the infarct volume, compared to the vehicle-injected mice ($n = 5$, infarct volume = $48.1 \pm 4.2\%$; Fig. 4A). The mortality rate in this group was 8.3% and 8.3% of mice were excluded for insufficient CBF reduction. There was no difference between treated and control mice. Similar to PcTX injection, the neuroprotective effect by bicarbonate was still present with delayed administration. For example,

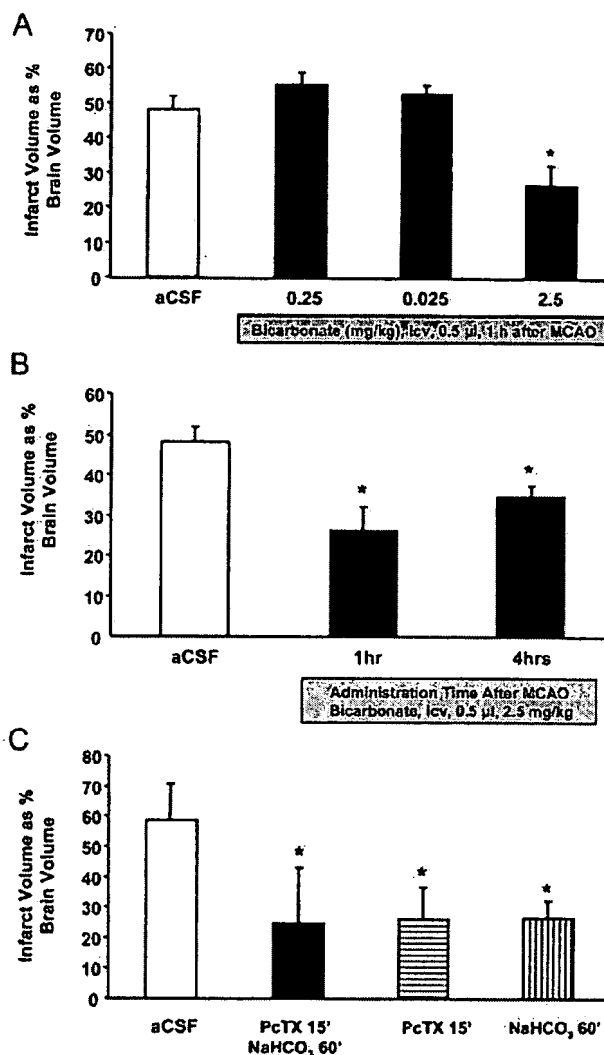


Fig. 4 (A) Concentration-dependent effect and (B) time window of efficacy of i.c.v. sodium bicarbonate; and (C) combined effect of i.c.v. PcTX with i.c.v. sodium bicarbonate on infarct volume induced by MCAO. Each column represents the mean \pm SD of the percentage of the infarct compared with the ipsilateral hemisphere. * $P < 0.05$ versus vehicle-treated group.

when bicarbonate (2.5 mg/kg) was administered 4 h after MCAO, significant protection was still present. The relative infarct volume was $48.9 \pm 3.8\%$ in aCSF-injected mice ($n = 5$), but reduced to $34.5 \pm 2.8\%$ in animals treated with bicarbonate 4 h after MCAO ($n = 6$; Fig. 4B). While 8.3% of mice died after surgery, 4.2% of mice were excluded for insufficient CBF reduction.

In contrast to i.c.v., i.v. injection of bicarbonate was not effective. The percentage of hemispheres infarcted were $60.44 \pm 13.0\%$ for saline, 61.51 ± 12.2 and $55.4 \pm 19.3\%$ for 2.5 and 5 mg/kg bicarbonate-treated mice, respectively ($n = 5$ for all groups, data not shown). The failure to observe the neuroprotective effect when bicarbonate was injected

i.v. may be explained by the finding that i.v. injection of bicarbonate is not effective in modifying brain pH. By contrast, when bicarbonate was i.c.v. administered 2 h after reperfusion, a marked and sustained increase in the brain pH value from 6.60 ± 0.60 to 7.25 ± 0.25 was observed ($n = 3$, Fig. 4B). That bicarbonate acts by attenuating activation of ASIC1a is supported by the observation that administration of bicarbonate (i.c.v., 0.5 μ l, 2.5 mg/kg) 60 min after MCAO in mice treated with PcTX 15 min (i.c.v., 0.5 μ l, 500 ng/ml) after MCAO does not induce a further reduction of the infarct volume (24.4 ± 18.5 ; $n = 5$) compared with the effect of PcTX alone (25.8 ± 10.7) or bicarbonate alone (26.2 ± 5.8 ; Fig. 4C).

Alteration of brain tissue pH after ischaemia

A prolonged neuroprotective time window with ASIC1a blockade suggests that there is a persistent acidosis in ischaemic brain, which activates the ASICs. We therefore used a fibre optic pH sensor to continuously measure brain pH in the ipsilateral and contralateral parietal cortex of mice immediately after the removal of the MCA suture, to determine the degree, distribution and the time course of acidosis following focal ischaemia. An increase of pH to 7.63 ± 0.11 was observed in this area during the first hour of reperfusion (2 h after MCAO). After this, pH dropped gradually and markedly acidic pH values were observed at

3 (6.69 ± 0.16) and 4 h (6.58 ± 0.07) after the MCAO (Table 1). No significant changes in pH values were observed in sham-operated animals; and the i.c.v. injection of PcTX, 3 h after MCAO, had no effect on pH changes (6.69 ± 0.16 vs. 6.78 ± 0.13 , Table 1).

To correlate pH values and ischaemic damage, mice were sacrificed 1, 2 and 4 h after reperfusion. The brains were quickly removed, cut into 1 mm thickness slices and stained with the vital dye TTC (Table 1). An alkaline pH was detected when the electrode tip was in uninfarcted brain i.e. TTC positive (red). As this region will eventually infarct, it is the penumbra area. When this area infarcts and becomes TTC negative (white), sustained acid pH values were observed.

Discussion

Despite many reports of pharmacological compounds showing significant neuroprotection in experimental stroke, no major clinical trials of a neuroprotectant have shown improved outcome. Up until now in the United States and in Europe the only currently approved medical treatment is the administration of tissue plasminogen activator within 3 h of stroke onset (Marler, 1995). One of the reasons for the lack of treatment success is the complexity of the mechanisms involved in ischaemic neuronal death and the fact that current available neuroprotective agents (e.g. NMDA receptor antagonists) have a short time window of effectiveness (Gladstone *et al.*, 2002). Our present studies suggest that ASICs may represent a better ischaemic target due to the long-term protective time window of ASIC blockade.

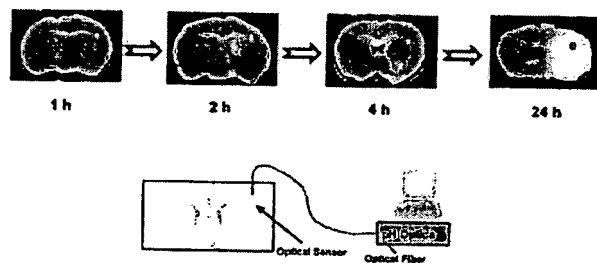
It has been known for several decades that acidosis occurs after ischaemia and that acidosis is associated with neuronal injury (Astrup *et al.*, 1977; Siesjo, 1992). Recent studies have reported that activation of the Ca^{2+} permeable ASIC1a plays a pivotal role in the development of acidosis-mediated ischaemic damage (Xiong *et al.*, 2004; Yermolaieva *et al.*, 2004). The importance in knowing the change in pH values with time after ischaemia resides in the fact that with this knowledge it is possible to precisely predict when and where ASICs might be activated. We demonstrated that, after ischaemia, pH is subjected to dynamic changes. Our experiments show that pH values change before the development of the ischaemic infarction, reaching values capable of activating ASIC1a at different times and different phases of injury.

In particular, as previously reported (Back *et al.*, 2000), in the penumbral region there is an initial pH alkalization while during the development of the ischaemic lesion, the core pH drops to values near 6.5. Such levels are sufficient to activate ASIC1a channels, which have a $\text{pH}_{0.5}$ at 6.2 (Escoubas *et al.*, 2000; Yermolaieva *et al.*, 2004).

The alkalosis phenomenon of the penumbra has been previously described as resulting from a reduction of lactate formation, the elevated phosphorylation of adenosine

Table 1 Effect of ischaemia on cerebral pH in sham-operated, vehicle-treated, PcTX-treated and sodium bicarbonate-treated animals

Time after MCAO (h)	Sham	Vehicle	PcTX	NaHCO_3
1	7.26 ± 0.05	7.16 ± 0.05	7.32 ± 0.04	7.26 ± 0.03
2	7.18 ± 0.11	7.63 ± 0.11	7.70 ± 0.03	7.41 ± 0.48
3	7.15 ± 0.07	6.69 ± 0.16	6.78 ± 0.13	6.60 ± 0.60
4	7.16 ± 0.03	6.58 ± 0.07	6.66 ± 0.09	7.25 ± 0.65



Values are mean \pm SE ($n = 3$ for each group). PcTX (0.5 μ l, 500 ng/ml) and sodium bicarbonate (0.5 μ l, 2.5 mg/kg) were i.c.v. injected 3 h after MCAO. Lower panel: corresponding time-dependent development of infarct in penumbra regions (mice sacrificed 1, 2, 4 and 24 h after reperfusion, 1 mm brain slices stained with the vital dye TTC). The yellow circle indicates the point at which the pH measurement was made.

nucleotides consuming H^+ , and acceptance of protons by the Krebs cycle (Back *et al.*, 2000). Nevertheless, the alkalosis in penumbra is only transient and, ~3 h following the induction of ischaemia, the pH in penumbra region drops to ~6.5, a level sufficient to activate ASIC1a. While $pH_{0.5}$ of ASIC1 is 6.2, the activation threshold of the channel is ~7.0 (Xiong *et al.*, 2004). Further, the induced channel current and the opening time are greater during ischaemia (Xiong *et al.*, 2004). As illustrated in Table 1, channel activation is expected to occur in the penumbra at 3 and 4 h after stroke induction as pH in the penumbra drops to 6.6–6.7. Importantly, this pH drop precedes the development of infarct in the penumbra.

Therefore the pH drop and activation of ASIC1a proceeds peripherally from the ischaemic core into the ischaemic penumbral region as infarction matures. This delayed acid expansion into the cortical penumbral region may explain the long neuroprotective window provided by ASIC1a blockade, and thus the protection observed with blockade, 5 h after stroke onset. The finding that PcTX is able to reduce the infarct volume even if administered after permanent occlusion of the middle cerebral artery indicates that the ASIC activation in the penumbra occurs in the absence of reperfusion. Furthermore, our finding that PcTX administration reduced the lesion for at least 7 days suggests that acute ASIC blockade does not simply delay the injury process.

The neuroprotection by bicarbonate and its time window of efficacy, also supports the central role of acidosis and ASICs in the evolution of infarction during the transition of the penumbra from alkalosis to acidosis.

Of particular clinical interest, the i.n. administration of PcTX was nearly as effective as PcTX administered i.c.v. The i.n. route is recognized as providing a method for bypassing the BBB and directly delivering therapeutic drugs to the CNS (Illum, 2000; Thorne and Frey, 2001). Drugs administered to the nasal cavity can pass through the olfactory and trigeminal nerves to reach various regions of the CNS. This method has already been used in experimental stroke (Liu *et al.*, 2001a, b) and in other neurodegenerative diseases (Capsoni *et al.*, 2002). Consequently, PcTX as well as other commonly used substances including oestrogen, cephalixin, dopamine, nerve growth factor and insulin-like growth factor-1 (Liu *et al.*, 2001a, b; Thorne and Frey, 2001) are able to reach the CNS through the nasal cavity. Since ischaemia represents a phenomenon that involves a complex pathogenic cascade of events which includes energy depletion, excitotoxicity, acidosis, and peri-infarct depolarization (Dirnagl *et al.*, 1999), an ideal treatment might need to make use of the combined therapy. Activation of ASIC1a and resultant membrane depolarization is expected to facilitate NMDA receptors activation (Wemmie *et al.*, 2002). On the other hand, it has been recently reported that the activity of ASIC1a following cerebral ischaemia is enhanced by the phosphorylation of Ser478 and Ser479 (Gao *et al.*, 2005). This phosphorylation

is catalysed by CaMKII activity, as a result of NMDA receptor activation (Gao *et al.*, 2005). Our data show that the combined therapy of ASIC1a blockade and NMDA receptor antagonism resulted in a further reduction of brain damage, as compared with the effects of the individual compounds, and also extended the therapeutic time window for NMDA blockade.

Together, these results and those we have previously reported (Xiong *et al.*, 2004) suggest that ASIC1a is a novel molecular and highly effective target involved in ischaemic brain injury.

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